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Inhibitory effects of (−)-epigallocatechin gallate on the life cycle of human immunodeficiency virus type 1 (HIV-1)

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Abstract

Epigallocatechin gallate (EGCg), the major tea catechin, is known as a potent anti-bacterial agent. In addition, anti-tumor promoting, anti-inflammatory, anti-oxidative and antiviral activities have been reported. In the present study, we investigated possible *anti*-human immunodeficiency virus type-1 (HIV-1) activity of EGCg and its mechanisms of action in the viral life cycle. EGCg impinges on each step of the HIV life cycle. Thus, destruction of the viral particles, viral attachment to cells, post-adsorption entry into cells, reverse transcription (RT), viral production from chronically-infected cells, and the level of expression of viral mRNA, were analyzed using T-lymphoid (H9) and monocytoid (THP-1) cell systems, and antiviral protease activity was measured using a cell-free assay. Inhibitory effects of EGCg on specific binding of the virions to the cellular surfaces and changes in the steady state viral regulation (mRNA expression) due to EGCg were not observed. However, EGCg had a destructive effect on the viral particles, and post-adsorption entry and RT in acutely infected monocytoid cells were significantly inhibited at concentrations of EGCg greater than 1 μM, and protease kinetics were suppressed at a concentration higher than 10 μM in the cell-free study. Viral production by THP-1 cells chronically-infected with HIV-1 was also inhibited in a dose-dependent manner and the inhibitory effect was enhanced by liposome modification of EGCg. As expected, increased viral mRNA production was observed in lipopolysaccharide (LPS)-activated chronically HIV-1-infected cells. This production was significantly inhibited by EGCg treatment of THP-1 cells. In contrast, production of HIV-1 viral mRNA in unstimulated or LPS-stimulated T-lymphoid cells (H9) was not inhibited by EGCg. *Anti*-HIV viral activity of EGCg may thus result from an interaction with several steps in the HIV-1 life cycle.

Keywords: HIV; Reverse transcriptase; Epigallocatechin gallate (EGCg); Viral protease

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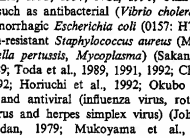
1. Introduction

Epigallocatechin gallate is one of the chemicals that can be extracted from tea. Tea families are classified by the procedures used to manufacture them from the leaves of *Camellia sinensis*. Polyphenol oxidase-inactivated, unfermented leaves are used to make green tea; black tea leaves are fermented thoroughly; and oolong tea leaves are incompletely fermented. Green tea contains 10–15% catechin, which includes several isomers, (−)-EGCg, (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC) and a little (+)-catechin. EGCg is approximately 50% of the total amount (Fig. 1). All of these catechins have a main structure of flavan-3-ols, and additional pyrogallol or galloyl groups that contribute to their biological activities. EGCg is the most potent compound of those isomers since it possesses both pyrogallol and galloyl moieties (Ikigai et al., 1990, 1993; Toda et al., 1990; Mukoyama et al., 1991; Nakayama et al., 1993).

Many unique anti-microbial activities (Hamilton-Miller, 1995, 1997; NCI, 1996; Yam et al., 1997) such as antibacterial (*Vibrio cholerae*, enterohemorrhagic *Escherichia coli* (0157: H7), methicillin-resistant *Staphylococcus aureus* (MRSA), *Bordetella pertussis*, *Mycoplasma*) (Sakanaka et al., 1989; Toda et al., 1989, 1991, 1992; Chosa et al., 1992; Horiuchi et al., 1992; Okubo et al., 1997), and antiviral (influenza virus, rotavirus, poliovirus and herpes simplex virus) (John and Mukundan, 1979; Mukoyama et al., 1991; Nakayama et al., 1993), antifungal (*Trichophyton*) (Okubo et al., 1991) and anti-toxin (cholera toxin, hemolysin) (Okubo et al., 1989; Ikigai et al., 1990; Toda et al., 1990, 1992) effects, were previously reported by us and others. The investigated bactericidal functions of EGCg are mainly dependent on charges in the bacterial membrane. Negatively charged EGCg is bound to the positively charged phospholipids of the membrane and causes damage to the lipid bilayer (Ikigai et al., 1993). The inhibitory effect of EGCg on infection by influenza virus was related to binding of EGCg to haemagglutinin (HA) spike proteins, major components of the receptor-binding sites of the virus and the resultant blocking of viral attachment to the receptors of target cells (Mukoyama et al., 1991). Furthermore, studies of anti-tumor effects have been progressing (Jankun et al., 1997; Yang, 1997). A recent study revealed that the anti-tumor effect correlates with inactivation of tumor-related proteases (Jankun et al., 1997). Another group reported inhibition of the induction of nitric oxide synthase (NOS) as an important factor in decreasing inflammation and inhibiting multiple stages of carcinogenesis (Lin and Lin, 1997). These mechanisms can be explained by decreased NOS activity and decreased protein levels of inducible NOS (iNOS) resulting from reduced expression of iNOS mRNA in lipopolysaccharide (LPS)-activated macrophages exposed to EGCg. Furthermore, the decreased amount of iNOS is mediated by blocking the signal transduction pathway of nuclear factor-κB (NF-κB). NF-κB is necessary for activation of the iNOS promoter. The consequences of these biochemical alterations reveal the underlying mechanisms associated with the anti-inflammatory, anti-carcinogenic, and radical-scavenging properties of EGCg. NF-κB is also a potent regulatory factor of HIV replication (Staal et al., 1990; Bachelerie et al., 1991; Riviere et al., 1991; Ross et al., 1991). In a related report with HIV, inhibition of RNA and DNA polymerases and thus inhibition of reverse transcription was observed in cell-free chemical assays (Nakane and Ono, 1990).

Thus EGCg, the major tea catechin, is not only known to be a potent anti-bacterial agent, but also has been reported to be antiviral, anti-tumor promoting, anti-inflammatory and anti-oxidative.



	MW
(-)-epicatechin (-EC): R_1=R_2=H	290
(-)-epigallocatechin gallate (-EGCg): R_1=OH, R_2=X	458

Fig. 1. Structure of EGCg and EC.

Accordingly, we recognize that EGCg has multiple functions, although these may be restricted by the tertiary structure of the target and result in non-specific activities. Our most recent bacteriological studies suggested that EGCg had some ability to enhance the biological effects of antibiotics and inhibited the emergence of drug resistance during combined antibiotic treatment (unpublished data). These actions may relate to the non-specific activities of EGCg since specific antimicrobial agents are capable of producing resistance in the long run.

Though we have investigated the effects of EGCg on several types of viruses and EGCg has produced beneficial results, detailed mechanisms of the antiviral activities of EGCg are still unclear. Therefore, we designed this study expecting that EGCg would have some inhibitory effects on the life cycle of HIV in vitro, and examined possible *anti*-HIV-1 activity of EGCg using two different host cell systems, T-lymphoid (H9) and monocytoid (THP-1) cell lines (Yamaguchi et al., 1997).

2. Materials and methods

2.1. Preparation of catechins

(−)-EC and (−)-EGCg were extracted and purified from green tea. Green tea leaves (*C. sinensis* L.) were suspended in hot water at the ratio of 100 g in 1000 ml, and a crude catechin mixture was extracted from the water-soluble fraction with chloroform and ethyl acetate. After concentration and lyophilization of the catechin mixture in the ethyl acetate soluble fraction, EC and EGCg were purified by high performance liquid chromatography using a Waters Prep PAC-500/C_{18} column (Millipore Corp., Bedford, MA), then equilibrated with tetrahydrofuran/acetone/H_2O.

2.2. EGCg-liposomes

Liposomes were constructed with 30% phosphatidylcholine (PC), 40% cholesterol, and 30% phosphatidylserine (PS). The lipids were dissolved in chloroform and stored at −120 °C until further use. Aliquots containing 5 μM lipids were prepared in glass tubes, and the chloroform was evaporated completely using nitrogen gas. After the sample was dried, the mixed lipids were suspended in 500 μl PBS using a glass rod and then mixed with 500 μl 20 mM EGCg in PBS. The solution was sonicated for 5 min at 20 W using the Ultrasonic Processor VC-50T (Sonics & Materials, Newtown, CT) and dialyzed against 10 l PBS in a porous membrane tube (MWCO 15000, Spectrum Medical Industries, Laguna Hills, CA) for 5 h at 4 °C (Ikigai et al., 1993). The concentration of the dialyzed EGCg-liposomes was calculated by comparing absorbance at 280 nm with a titration curve obtained using unconjugated EGCg and liposomes.

2.3. Cells and virus

T-lymphoid H9 (HUT78) cells were obtained from Gallo (National Cancer Institute, Bethesda, MD) (Gazdar et al., 1980; Popovic et al., 1984). The THP-1 mature monocytic cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). These cell cultures were maintained in RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), penicillin (250 units/ml), streptomycin (250 μg/ml), 2 mM L-glutamine, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (complete medium) at 37 °C in 5% CO_2.

MAGIC-5 cells were derived from HeLa-CD4-LTR-β-gal (MAGI) cells, and express both the CD4 and CCR5 receptors (Hachiya et al., 2001). MAGI cells were engineered to express the β-chemokine receptor CCR5 by transfecting MAGI cells with the expression vector *pEFBOSb-srHuCCR5*, in which expression of human CCR5 is promoted by the elongation factor-1α promoter, and followed by selection with blasticidine. The cell culture was maintained in D-MEM (GIBCOBRL) complete medium (5% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES buffer) supplemented with 0.2 mg/ml

G148 (Sigma Chemical Co.), 50 units/ml hygromycin (Sigma Chemical Co.) and 1 μg/ml blasticidin S (Funakoshi Chemical Co., Tokyo, Japan) at 37 °C in 5% CO_2.

Human monocyte-derived macrophages (MDM) were obtained from healthy HIV-1-seronegative donors. Briefly, peripheral blood mononuclear cells (PBMC) were separated over Ficoll-Hypaque gradients (Ficoll-Paque PLUS; Amersham, Pharmacia Biotec, Tokyo, Japan), and the PBMC were incubated for adhesion onto the plastic of a tissue culture dish in RPMI-1640 medium for 2 h. Adherent cells were cultured in Macrophage-SFM medium (GIBCO BRL), supplemented with 10% human AB serum, 10 units/ml of monocyte-colony stimulation factor (M-CSF; Genetics Institute, Cambridge, MA), penicillin (250 units/ml), streptomycin (250 μg/ml) at the concentration of 1×10^6 cells per well in 48-well plates and progressively allowed to differentiate into mature macrophages for 7 days at 37 °C in 5% CO_2. The purity of all cultures using this method is typically > 98% MDM as determined by fluorescence-activated cell sorter analysis using an *anti*-CD14 monoclonal antibody (DAKO, Kyoto, Japan).

HIV-1_{NRB} was obtained from Gallo (National Cancer Institute) (Gallo et al., 1984). HIV-1_{MN} was obtained from the AIDS Research and Reference Reagent Bank (Division of AIDS, NIAID, NIH; contributed by Gallo) (Gallo et al., 1984; Shaw et al., 1984). Virus stocks of HIV-1 were prepared from filtered cell-free supernatants of chronically-infected H9 cultures by the shaking method described previously (Chambers et al., 1991).

2.4. Cytotoxicity of EGCg and EC

H9 or THP-1 cells (2×10^5 cells/ml) were incubated with appropriate concentrations of EGCg or EC in 2 ml for 2 days at 37 °C. The cells were washed twice with 10 ml of RPMI-1640 medium and resuspended in 2 ml of complete medium. Aliquots containing 200 μl of each cell solution were transferred to 96-well plates and incubated with 20 μl *alamarBlue* (SEROTEC UK, Kidlington, Oxford OX5 1JE, England) for 8 h at 37 °C. The absorbance was measured spectrophotometrically at wavelengths of 570 nm for analysis, and 600 nm for background. The median lethal dose (LD_{50}) was analyzed according to the Manufacturer's instructions.

2.5. Direct effect of EGCg on the viral particle

MAGIC-5 cells were plated in 96-well plates at 4×10^4 cells per well in 100 μl of complete D-MEM medium the day before infection. HIV-1_{NRB} (50 ng p24 antigen in 1 ml) were mixed with indicated concentrations of EGCg for 0, 1, or 3 h at 37 °C in 1 ml of complete D-MEM medium. The medium in 98-well plates was removed from each well, and 100 μl of the EGCg-treated virus solution was added and incubated for 2 h. The cells were then washed twice with D-MEM and incubated with 200 μl of complete D-MEM medium for 2 days at 37 °C in a 5% CO_2 incubator. After incubation, the medium containing virus was removed, and the cells were fixed using 50 μl of fixation solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min and then washed with PBS. The fixed cells were incubated with 100 μl of staining solution [4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$ and 0.4 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, Sigma Chemical Co.) in PBS] for 60 min at 37 °C. The staining was stopped by removing the staining solution and washing twice with PBS. The number of blue stained infected cells in each well was counted using a light microscope.

2.6. Binding of viral particles to cellular surfaces

Binding experiments were performed using two different methods such that either cells (H9 or THP-1: 4×10^6 cells/ml) or viruses (HIV-1_{NRB}: 50 ng of p24 antigen/ml) were treated with the indicated EGCg concentrations for 30 min at 37 °C in 500 μl of complete medium. The EGCg-treated cells were then washed gently once with 10 ml RPMI-1640 medium (inverted 10 times in 15 ml of conical tube) and mixed with 1 ml of fresh virus containing 25 ng of p24 antigen/ml (Mon-

tefiori et al., 1993). Binding reversibility experiments were performed by washing several times. The EGCg-treated viruses were then mixed with 500 μl of H9 or THP-1 cells (4×10^6 cells/ml). These tubes were incubated for 2 h at 37 °C, then the cells were washed once with 10 ml of cold PBS. The cell pellets were lysed in 500 μl of 0.5% Triton-X100 in PBS, the debris was removed by brief centrifugation, and the p24 antigen concentration was quantitated using a commercial enzyme immunoassay (EIA; Abbott Laboratories, North Chicago, IL) according to the Manufacturer's instructions.

2.7. Efficiency of acute infection

H9, THP-1 cells (2×10^5 cells/ml) or MDM (1×10^6 cells/well in 24-well plate) were treated with the indicated EGCg concentrations for 3 days at 37 °C before virus exposure. The cells were then washed three times with of RPMI-1640 medium, to remove external EGCg, and exposed to HIV-1_{IIIB} (25 ng/ml p24 antigen in 1 ml) in complete medium at 37 °C for 2 h. After virus adsorption, the cells were washed twice with RPMI-1640 medium and resuspended in fresh complete medium containing the indicated concentrations of EGCg. After incubation for an additional 48 h at 37 °C, the cells were harvested for DNA extraction. The infected cells were washed twice with PBS (phosphate-buffered saline, GIBCOBRL) and then suspended in lysis buffer (10 mM Tris–HCl [pH 8.4], 50 mM KCl, gelatin [100 μg/ml], 2.5 mM $MgCl_2$, 0.45% IGEPAL CA-630, 0.45% polyoxyethylene (20) sorbitan monolaurate, proteinase K [60 μg/ml]) at a concentration of 1×10^7 cells/ml. After incubation of the mixtures at 56 °C for 1 h, proteinase K was inactivated by incubation at 95 °C for 10 min. The HIV-1 viral DNA (*gag* gene) was amplified and quantitated using a commercial competitive PCR assay (Maxim Biotech, Inc., San Francisco, CA) according to the Manufacturer's instructions. Standard 2% agarose gel electrophoresis was performed using 10 μl of amplified samples, and the bands were visualized by ethidium bromide (EtBr) staining.

2.8. Virual production from chronicall infected cells

H9 or THP-1 cells chronically infected with HIV-1_{IIIB} (IIIB/H9, IIIB/THP-1, respectively) or HIV-1_{MN} (MN/H9) were maintained in continuous culture for at least 3 months after initial infection. The stability of viral production in the cultures was monitored by sequential p24 antigen measurements. These infected cells were treated with the indicated concentrations of EGCg or EGCg-liposomes at 2×10^5 cells/ml in 2 ml complete medium using 16-mm 24-well plates (Corning Costar Corp., Cambridge, MA). Half of the culture medium was exchanged every 3 days while maintaining the indicated drug concentrations. The supernatant p24 antigen level and the cell viability were observed 10 days after initiation of treatments.

2.9. Measurement of the expression of viral mRNA in chronically-infected cells

Chronically infected IIIB/H9, IIIB/THP-1 cells (2×10^5 cells/ml) were treated with complete medium containing 40 μM EGCg for 2 days, and then stimulated with 2.5 μg/ml LPS (lipopolysaccharide, *E. coli* 0127:B8, Sigma Chemical Co.). Eight hours after stimulation, the cells were harvested for RNA extraction and total cellular RNA was prepared using *TRI ZOL Reagent* (GIBCOBRL) according to the Manufacturer's instructions (Chomczynski, 1993). The prepared total RNA pellet was dissolved in 50 μl of diethylpyrocarbonate (DEPC) in water. The HIV-1 envelope (env) mRNA and human β-actin mRNA, the constitutively-transcribed control gene, were amplified from total RNA using reverse transcription and the polymerase chain reaction (RT/PCR). The 50 μl reaction volume contained 100 ng of total RNA, 1 × EZ Buffer (50 mM Bicine, 115 mM potassium acetate, 8% (w/v) glycerol, [pH 8.2]; Perkin Elmer), 2.5 mM $Mn(OAc)_2$ solution, 300 μM each dATP, dGTP, dCTP, dTTP, 0.5 μM each SK68 primer (AGC AGC AGG AAG CAC TAT GG, 7801–7820, env region), SK69 primer (CCA GAC TGT GAG TTG CAA CAG, 7922–7942, env region) (Ou et

al., 1988; Zimmerman et al., 1996), or 0.5 μM each β-actin primer pair (primer 1: TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA, 509–538; primer 2: CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG, 1140–1169) and 5 U of rTth DNA polymerase (Perkin Elmer). Reverse transcription was performed at 60 °C for 60 min. Subsequent DNA amplification was performed using an initial step of 96 °C for 1 min and a two-step amplification, 2 cycles of 94 °C for 1 min and 60 °C for 4 min, 35 cycles of 94 °C for 1 min and 60 °C for 2 min 30 s, then ending with 1 cycle of 72 °C for 8 min. Standard 2% agarose gel electrophoresis was performed using 10 μl of amplified samples and EtBr-stained band intensities were analyzed using the Kodak Digital Science 1D Image Analysis System.

2.10. Measurement of HIV-1 protease kinetics

Indicated concentrations of EGCg or EC were incubated with 1.4 μg of recombinant HIV-1 protease (storage in 0.1 M sodium acetate, [pH 5.5], 10% (v/v) glycerol, and 5% (v/v) ethylene glycol, BACHEM Bioscience Inc., Horizon Drive, PA) (Nutt et al., 1988; Navia et al., 1989) in 400 μl of reaction buffer (50 mM sodium acetate, [pH 4.9], 200 mM NaCl, 5 mM DTT, 10% (v/v) glycerol) at 37 °C for 10 min, and then mixed with 10 μl of the 1 mg/ml quenched fluorogenic substrate Dabcyl-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS (4-(4-dimethylaminophenylazo)benzoyl-γ-aminobutyric acid-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid, BACHEM Bioscience Inc.) (Matayoshi et al., 1990). The mixed solution was transferred to 96-well Fluoroplates (Labsystems, Sorvaajankatu, Helsinki, Finland) at 200 μl per well and the emitted fluorescence intensity was monitored kinetically using a fluorescence scanner (Fluoroskan II, Labsystems) at 37 °C using filters of 355 nm for excitation and 460 nm for emission. The quenched fluorogenic substrate method using EDANS was selected because of the technical difficulties associated with the use of the anthranilyl-HIV protease substrate (Abz-Thr-Ile-Nle-*p*-nitro-Phe-Gln-Arg-NH_2), i.e. the very narrow range of acceptable absorbance measurements and the specified wavelength, 300 nm, which is close to the peak absorption spectrum of EGCg (285 nm). When quenched, the intrinsic fluorescence of EDANS is reduced by intra-molecular resonance energy transfer (RET) to the Dabcyl group, and when released by protease hydrolysis, the increase in fluorescence intensity is linearly related to the rate of hydrolysis of the fluorogenic substrate. The peak of the fluorescence spectrum of free EDANS is 472 nm.

2.11. Comparison of the effect of EGCg and EC on chronically-infected cells

Chronically infected IIIB/THP-1 cells were incubated with indicated concentration of EC, EGCg, AZT (3′-azido-3′-deoxythymidine, Sigma Chemical Co.) or Indinavir (MK-639 INDINVIR; contributed by Merck Research laboratories) at 2×10^5 cells/ml in 2 ml for 14 days. AZT and Indinavir were used as control inhibitors. Half of the culture medium was exchanged with complete medium while maintaining the indicated drug concentrations, and p24 antigen levels were monitored every 3 days.

2.12. Statistical analysis

Data were expressed as mean ± SD. Experimental groups were compared to control groups using the single-factor analysis of variance (ANOVA). If statistical significance ($P < 0.05$) was reached by ANOVA, post hoc comparisons of the means were performed using the Tukey–Kramer test (Kramer, 1956). A P value less than or equal to 0.05 was considered significant.

3. Results

In preliminary experiments we established the effects of EGCg on uninfected H9 and THP-1 cells with respect to cell viability and growth. We compared results obtained using the methods of trypan blue dye exclusion, *alamarBlue* dye reduction and microscopic observation of cell number increase. These initial assays produced concordant results so that trypan blue dye exclusion was used

Table 1
Direct effect of EGCg on the virus particle

Incubation time (h)	Infected cell number/well (% inhibition) [a]			
	EGCg concentration (μM)			
	0	1	10	100
0	181.2 ± 17.7	182.8 ± 18.7 (0)	182.4 ± 35.0 (0)	4.0 ± 1.9 (97.8)
1	177.8 ± 34.9	176.2 ± 48.3 (0.9)	59.4 ± 18.9 (66.5)	3.6 ± 1.7 (98.0)
3	93.6 ± 18.2	56.2 ± 9.6 (40.0)	21.2 ± 5.8 (93.8)	1.0 ± 1.0 (98.9)

MAGIC-5 cells were plated in 96-well plates and incubated with EGCg treated HIV-1_{IIIB} for 2 h and then the cells were incubated for 2 days. The infected cells were stained and counted by by microscopic observation.

[a] Each value represents the mean ± S.D. from three separate experiments, each performed in triplicate. The results of % inhibition were derived from comparing with untreated control (0 μM) for each incubation time.

for the quantitative evaluation of toxity in subsequent virological experiments. The LD_{50} of EGCg (174.8 ± 16.7 μM H9; 440.3 ± 12.4 μM THP-1) and EC (1990.7 ± 43.8 μM H9; 2828.3 ± 220.0 μM THP-1) were analyzed using *alamarBlue* dye reduction, and no significant differences in cell viability were observed throughout these experiments at any dose of EGCg tested.

3.1. Direct effect of EGCg on viral particles

The capacity of EGCg to destroy virus was measured following different times of incubation, as indicated (Table 1). The number of infected MAGIC5 cells indicated that the ability of infection of virus particles in medium containing EGCg. Dose- and incubation time-dependent inhibition of virus infection of the MAGIC-5 cells were observed, however, infected cell number at 100 μM was strongly decreased for all incubation times tested. Although, this general tendency was also observed for incubation times longer than 3 h, the data are not shown since the control (0 μM) could not be validated.

3.2. Effect of EGCg on the attachment of viral particles to the surface of cells

Binding experiments were performed using two different methods to clarify whether EGCg binds to cellular or viral surfaces when blocking the attachment of the viral particles to cellular surfaces. Pretreatment experiments were performed such that cells were incubated with EGCg for 2 h, unbound EGCg was removed, and the cells were incubated with virus. For the second method, viruses were preincubated with EGCg and subsequently mixed with cells. The later experiments evaluated whether EGCg binds to both viral particles and cellular surfaces. The virus stock prepared by the shaking method contains virion-associated and -free p24 antigens; the percentage is 85–90 and 10–15%, respectively. In the presence of control medium, approximately 1–2% of the total viral p24 became cell associated. Viral attachment was inhibited about 20% at 100 μM EGCg in both experiments, and no significant differences were observed between cell types, H9 and THP-1 (Fig. 2A). At concentrations less than 10 μM, EGCg had no influence on viral binding to cellular surfaces. These results suggest that a high concentration of EGCg is necessary for EGCg to bind to cellular surfaces and inhibit viral attachment. Furthermore, reversibility of the blocking of viral attachment was analyzed by washing the cells several times after EGCg treatment. Recovery of viral-binding ability was observed after more than two washes (Fig. 2B).

3.3. Effect of EGCg on post-adsorption entry and reverse transcription of viral DNA in acutely-infected cells

H9 cells or THP-1 cells were treated with EGCg for 3 days at the indicated concentrations. The cells were washed to remove EGCg in the medium

and on the cell surfaces, and the cells were exposed to HIV-1_{IIIB} or HIV-1_{MN}. The pretreatment was performed to allow uptake of EGCg by the target cells before viral entry, and to avoid the influence of a direct effect of EGCg on the viral particles or attachment. The period of 48 h after the initiation of infection was selected for measuring viral DNA synthesis resulting from first-round infection. This time point is too early for significant second-round infection events to have occurred in this system. Viral DNA in whole-cell lysates was analyzed using a competitive PCR method. A significant dose-dependent inhibition of IIIB viral DNA synthesis was observed for monocytoid cells (THP-1) and MDM, with approximately 10-fold inhibition for concentrations of 1 and 10 μM, respectively, whereas no significant inhibitory effect was observed for H9 cells infected by either IIIB or MN viruses (Fig. 3).

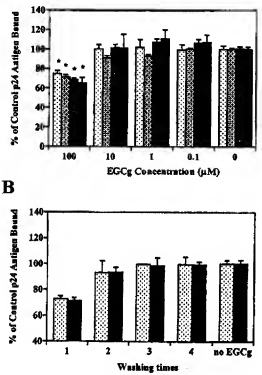


Fig. 2. (A) Attachment of viral particles to H9 or THP-1 cells in the presence of EGCg. Cells or virus were treated with indicated concentrations of EGCg. The ECGg-treated cells were then washed once and incubated with a constant amount of HIV-1_{IIIB} (cell pretreatment experiment). The EGCg-treated viruses were then mixed with a constant amount of cells without washing (virus pretreatment experiment). Bound virus was determined by p24 EIA after washing and detergent solubilization.▨, EGCg-pretreated H9 cells plus virus; ▩, EGCg-pretreated THP-1 cells plus virus; ▮, EGCg-pretreated virus plus H9 cells; ■, EGCg-pretreated virus plus THP-1 cells. (B) Reversibility of the interference of EGCg on the binding of virus to cell surfaces. The THP-1 cells were washed several times after treatment with 100 μM EGCg, and then washed cells were incubated with virus. Bound virus was determined using the method described above. In (A) and (B), bars represent the mean of three experiments, each performed in duplicate, while the error bars represent 1 S.D.; $^*P < 0.01$.

3.4. Effect of EGCg on chronically-infected cells

Chronically HIV-1-infected IIIB/H9, MN/H9 and IIIB/THP-1 cells were incubated with indicated concentrations of EGCg, and measurement of supernatant p24 antigen levels and the cell viability were performed on day 10 after initial treatment. A dose-dependent inhibition of viral production from chronically infected monocytoid cells (IIIB/THP-1) was observed (Fig. 4A). The highest EGCg concentration inhibited virus production by approximately 80% compared to untreated cultures. In contrast, no reduction in viral production at any concentration was observed for chronically infected T-lymphoid cells (IIIB/H9, MN/H9).

3.5. Effect of EGCg-liposomes on IIIB/THP-1 cells

PS-rich liposomes containing EGCg were selected for this study, since EGCg damages PC-rich liposomal membranes, due to catechin-mediated aggregation of the liposome related to their charge. Even this component had limitations related to EGCg concentration due to the aggregation. Therefore, we used liposomes with less than 40 μM EGCg in this experiment. These experiments were performed to examine the potency of liposomes in promoting intracellular uptake of EGCg and reducing the toxicity of this drug. EGCg-liposomes or unmodified EGCg were

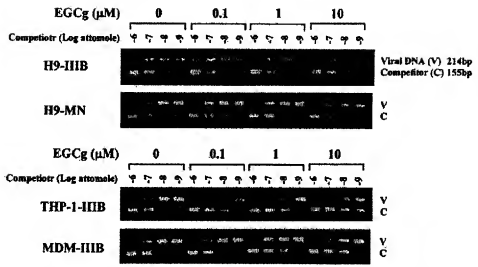


Fig. 3. HIV DNA production by H9, THP-1 cells or MDM infected with HIV-1_{IIIB} or HIV-1_{MN}. The cells were preincubated with EGCg for 3 days at the indicated concentrations, washed well and infected with virus for 2 h in the absence of EGCg, then cultured in the presence of EGCg for 48 h. Viral DNA was measured using competitive PCR. The EtBr-stained DNA bands are from a representative agarose gel of the PCR products.

incubated with chronically HIV-1-infected IIIB/THP-1 cells for 10 days and viral production was measured (Fig. 4B). The EGCg-liposomes were approximately 10–25% more effective at promoting intracellular uptake of EGCg than unmodified EGCg at each concentration tested. Throughout these experiments, no significant differences in cell viability or aggregation of liposomes in the medium were observed.

3.6. Effect of EGCg on the expression of viral mRNA in chronically-infected cells

Steady state or LPS-stimulated mRNA levels of chronically HIV-1-infected cells were analyzed by RT–PCR using an *env* gene-specific primer pair (Fig. 5). A β-actin primer pair was used as a measure of constitutive transcription and relative intensities of the HIV-1 env and β-actin mRNA bands were quantified using image analysis as described in Section 2. Steady state viral production from IIIB/THP-1 cells was relatively lower than that of IIIB/H9 cells as was observed for p24 antigen production by the cells. At steady state, no significant inhibition of viral mRNA expression was observed in either of the EGCg-treated chronically infected cell types, as compared to untreated controls. The expression of viral mRNA was increased by approximately 40–50% by LPS treatment in the absence of EGCg in both IIIB/H9 cells and IIIB/THP-1 cells. This LPS-stimulated increase was inhibited in IIIB/THP-1 cells, but not in IIIB/H9 cells, in the presence of EGCg.

3.7. Effect of EGCg on HIV-1 protease kinetics

EGCg at relatively high concentrations inhibited the protease activity in a dose-dependent manner (Fig. 6). Inhibition was observed at a remarkably early stage of the reaction and the concentrations required for continuous inhibition in this assay were greater than 10 μM (results not shown). The maximum percentages of inhibition

by 100 and 50 μM were 65 and 30%, respectively at 10 min after initial treatment. To clarify the importance of EGCg among the chatechin isomers, the effects of EC, one of the catechin structural analogs lacking galloyl, on protease kinetics was observed. Weak inhibition of protease kinetics at early stages of the reaction and no continous inhibition were observed with 100 μM EC.

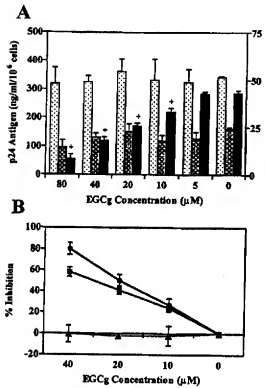


Fig. 4. (A) Supernatant p24 production by H9 or THP-1 cells chronically infected with HIV-1_{IIIB} or HIV-1_{MN}. Measurement of supernatant p24 antigen levels was performed on day 10 after initial treatment. The p24 antigen/cell number values for IIIB/H9 and MN/H9 are indicated using the left scale, IIIB/THP-1 values are indicated by the right scale. ▯, IIIB/H9; ▮, MN/H9; ■, IIIB/THP-1. The bars represent the mean of three experiments, each performed in duplicate, while the error bars represent 1 S.D.; $^*P < 0.01$. (B) Effect of liposome-treated EGCg on supernatant p24 production from THP-1 cells chronically infected with HIV-1_{IIIB}. EGCg-liposomes or unmodified EGCg were incubated with IIIB/THP-1 cells for 10 days and virus production was analyzed using a p24 antigen EIA. (■), EGCg; (●), EGCg-liposome; (▲), liposome control. Each point represents the mean of three experiments, each performed in duplicate, while the error bars represent 1 S.D.; $P < 0.05$.

3.8. Comparison of the effects of EGCg and EC on viral production from chronically-infected IIIB/THP-1 cells

The biological activity of EGCg on viral production in chronically infected monocytoid cells was compared with the biological activity of EC, AZT or Indinavir (Fig. 7). The concentrations of both 100 nM AZT and 1 μM Indinavir were approximately equal to 50 IC_{50} (50% inhibitory concentration) of each (Witvrouw et al., 2000). The inhibitory effect of EGCg on viral production began on treatment day 7, and p24 antigen levels decreased until they were 80–90% below those of untreated medium controls. By contrast, approximately 40% inhibition by EC of viral production in chronically infected cells was observed. The explanation for inhibition of progeny viral production with 100 nM AZT is that it may possess a post-integrational mode of action and may be able to complex with newly formed p66/51 to interfere with proper assembly of progeny HIV-1 at the cell membrane. The inhibition was observed in long-term experiments and not in short-term experiments (Rooke et al., 1990; Coates et al., 1992).

4. Discussion

The current studies analyzed possible *anti*-HIV activities of EGCg including destruction of the virion, obstruction of viral attachment to the target cells, and several anti-enzymatic effects in the viral life cycle.

EGCg destroyed virions in a dose- and incubation time-dependent manner. Virion destruction can be explained by EGCg binding to the surface of the viral envelope and destroying it by a method dependent on deformation of the phospholipids in a manner similar to polymixin B (PXB) effects on bacterial membranes (Ikigai et al., 1993). Inhibition of influenza virus adsorption to Madin-Darby canine kidney (MDCK) cells and

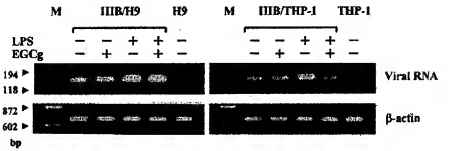


Fig. 5. Expression of viral env mRNA in H9 or THP-1 cells chronically infected with HIV-1_{IIIB} at steady state or LPS-stimulated state. Chronically HIV-1-infected IIIB/H9 cells or IIIB/THP-1 cells were treated with EGCg for 2 days, then the cells were stimulated with LPS. After an 8 h incubation in the presence of EGCg, the stimulated cells were harvested and the expression of the env mRNA and β-actin mRNA was analyzed using an RT–PCR method. Standard 2% agarose gel electrophoresis was performed using the PCR products, and DNA was stained with EtBr. M, ΦX174/HaeIII digest maker.

chicken erythrocytes (CRBC) by EGCg was previously reported by our laboratory (Nakayama et al., 1993). EGCg quickly bound to the virus and agglutinated it at the same potencies as specific antibodies for the virus, and blocking of viral adsorption to MDCK cellular surfaces was observed by electron microscopy and scanning electron microscopy. The haemagglutinin (HA) spike contains the principal glycoproteins used for adsorption and haemagglutination. Inhibition of these functions was achieved by binding of the EGCg molecule to that spike. Generally, viral envelopes are composed of cellular phospholipids components and HIV is not an exception to this. We performed a binding study to determine if EGCg might bind to glycoprotein (gp) 120 or gp41 as targets, or behave like phosphorothioate oligonucleotide polyanions, e.g. dextran sulfate or sulfated polysaccharides, and non-specifically interfere with viral adsorption (Mitsuya et al., 1988; Witvrouw and De Clercq, 1997; Yamaguchi et al., 1997). This experiment was distinguished from the virus-destruction experiment due to the shorter incubation time. Although EGCg has a negative charge and is capable of non-specifically binding to the viral envelope glycoproteins or specifically binding to viral binding receptors on the cell surface, like other negatively charged polymers including phosphorothioate oligonucleotides (Stein et al., 1991; Yamaguchi et al., 1997), no significant interference with viral adsorption was observed in this HIV study. We only observed reversible interference of viral attachment to the cellular membrane at the highest concentration of EGCg. The interference was not very strong, and it may have been caused simply by physically blocking the cell surface because washing the cells several times was enough to recover the virus-cell

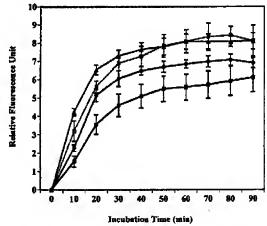


Fig. 6. Measurement of HIV-1 protease kinetics using a quenched fluorogenic substrate. The indicated concentrations of EGCg or EC were incubated with recombinant HIV-1 protease for 10 min and then mixed with quenched fluorogenic substrate. The emitted fluorescence intensity was monitored kinetically using a fluorescence scanner. (■), EGCg 100 μM; (●), EGCg 10 μM; (□), EC 100 μM; (▲), untreated control. Each point represents the mean of three experiments, each performed in duplicate, while the error bars represent 1 S.D.; $P < 0.05$.

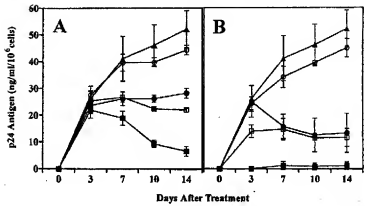


Fig. 7. Comparison of the effects of EGCg and EC on chronically-HIV-1_{IIIB}-infected THP-1 cells. Chronically infected IIIB/THP-1 cells were treated with the indicated concentrations of EGCg, EC, AZT or Indinavir and supernatant p24 production was monitored continually for the following 14 days. (A) (■), EGCg 50 μM; (□), EGCg 25 μM; (●), EC 50 μM; (○), EC 25 μM; (▲), untreated control. (B) (■), Indinavir 1 μM; (□), Indinavir 100 nM; (●), AZT 100 nM; (○), AZT 10 nM; (▲), untreated control. Each point represents the mean of three experiments, each performed in triplicate, while the error bars represent 1 standard deviation; $P < 0.05$.

binding ability. The strongly decreased infected cell number at 100 μM at all incubation times, observed in the virion-destruction experiment may also be due to additional physically blocking the cell surface. Despite the fact that both influenza virus and HIV have glycoproteins in their envelopes that mediate attachment to target cells, EGCg had no effects on HIV. This is thought to be due to the differences in the charge intensity, the tertiary structure and the amount of glycoside at the receptor binding region. Interference of this attachment and the low LD_{50} of EGCg for H9 cells made if necessary for us to design subsequent long-term incubation experiments in which we selected EGCg concentrations below 100 μM which do not interfere with viral attachment and do not decrease cell viability.

Inhibition of several DNA or RNA polymerases by catechin isomers has been reported by others (Nakane and Ono, 1990). The IC_{50} was approximately 25 nM and the level of inhibitory kinetics for HIV–RT was 5–50 fold higher than for other polymerases, e.g. DNA polymerase α, β, γ and *E. coli* RNA polymerase. Therefore, we investigated the RT inhibitory effects of EGCg on acute infection in vitro at post-adsorption entry and RT. These experiments were designed to avoid the possible inhibition by EGCg of viral adsorption, described above, by preloading cells with EGCg, then removing extracellular EGCg that may have adhered to the cell surface during the cell adsorption and entry steps. Two representatives of T-cell tropic viruses, IIIB and MN, were selected for infection of T-lymphoid cells to compare the effects of EGCg on strains that have different characteristics of infectivity, pathogenicity and level of viral propagation. In both cases, no effect of EGCg was seen. However, when monocytoid cells or MDM were infected with IIIB and treated with EGCg reduced HIV DNA levels were observed at relatively low concentrations of EGCg and in a dose-dependent manner. Although these infection experiments included several steps of the viral life cycle from post-adsorption to integration, the hypothesis that the effect of EGCg is due to RT inhibition is the most reliable hypothesis based on the results of the chemical assay.

In chronically HIV-1-infected cells, viral production in EGCg-treated monocytoid cells (IIIB/THP-1) was diminished, as compared to no change in viral production in EGCg-treated T-lymphoid cells (MN/H9, IIIB/H9). EGCg inhibited both viral infection and production only in the monocytoid cells; therefore, we suspect that the effects were dependent upon the characteristic

activity of the monocytoid cells, which is phagocytosis, and that the concentration of EGCg in the cells contributed to the inhibition. Liposome-mediated strategies for HIV infection, such as liposome-encapsulated RT inhibitors, protease inhibitors, anti-sense oligonucleotides and immunoliposomes for targeted therapies, have been evaluated (Szebeni et al., 1990; Flasher et al., 1994; Schreier et al., 1994; Zelphati et al., 1994; Dipali et al., 1996; Pretzer et al., 1997). The superiority of liposomal modification is that it enables the use of agents that focus on functions supporting cellular uptake while reducing generalized cytotoxicity of the drugs. We designed EGCg-encapsulated liposomes to clarify whether the internal concentration of EGCg in the cells relates to the inhibitory effects and expected to find a high efficiency of cellular uptake because inhibition of viral production from chronically infected cells required a higher concentration of EGCg than inhibition of the post-adsorption entry and RT steps. According to expectation, the liposome-encapsulated EGCg enhanced by approximately 10–25% the inhibitory effects of EGCg on viral production in monocytoid cells.

Inhibition of NO production by EGCg is mediated by blocking the signal transduction pathway for the activation of the iNOS promotor by NF-κB in LPS-stimulated macrophages, as reported by others (Lin and Lin, 1997). It is well known that NF-κB is also a potent regulatory factor for HIV replication (Staal et al., 1990; Riviere et al., 1991; Ross et al., 1991). Consequently, we examined whether EGCg down-regulated the production of HIV viral mRNA at both a steady or LPS-stimulated state. We chose not to measure steady or LPS-stimulated state mRNA levels during acute infection because of the difficulty in distinguishing among the inhibition of new rounds of infection, changes in the pattern of RNA transcription (from multiply- to single- or non-spliced), and changes in total level of mRNA per cell. At steady state, EGCg had no inhibitory effects on viral mRNA expression in either of the chronically infected cells (IIIB/H9, IIIB/THP-1). However, the increased viral mRNA production induced in IIIB/THP-1 cells by LPS stimulation was inhibited in the presence of EGCg. Production of viral mRNA in EGCg-treated, LPS-stimulated IIIB/H9 cells was unaffected. The possibility that LPS destruction of the LPS receptor or interference with LPS-receptor binding may have occurred was deemed to be negligible since viral production was significantly enhanced by LPS stimulation of IIIB/H9 cells in the presence of EGCg, and this was not observed at steady state when the NF-κB pathway was not activated. These data suggest that inhibition of viral mRNA production was related to the NF-κB pathway and that EGCg may not have as strong of a direct, down-regulatory effect on the HIV-1 promoter, which controls viral gene regulation, as stimulation of a member of the steroid hormone receptor superfamily (Ladias, 1994; Yamaguchi et al., 1994). It should be pointed out that the effects of EGCg were only observed in chronically infected monocytoid cells both in these studies and in studies by others where EGCg decreased NOS induction in macrophages (Lin and Lin, 1997). These findings of selective inhibition may be explained by continuous activation of NF-κB is required for the production of HIV-1 in monocytic cells but not in T cells (Jacqué et al., 1996).

The assertion that EGCg is the strongest biologically active form of the catechin isomers is due to the fact that it contains both of the pyrogallol and galloyl groups, and that EGCg treatment is beneficial in several types of bacterial infection as previously reported (Ikigai et al., 1990, 1993; Toda et al., 1990; Mukoyama et al., 1991; Nakayama et al., 1993). Potency of the galloyl group was addressed in the previous studies. Consequently, in these studies, we clarified whether the biological activities of EGCg against HIV were also dependent on the existence of the galloyl group by comparing EGCg with EC, which has no galloyl group. The anti-protease activity of EGCg was analyzed using computer modeling, since the function of a protease is important in the HIV life cycle. It was determined that EGCg inhibited urokinase (uPA) activitiy (Jankun et al., 1997). Though uPA is a serine proteolytic enzyme and the HIV-1 protease is an aspartyl proteolytic enzyme (Navia et al., 1989), we considered that the effect of EGCg on HIV-1 protease was worth analyzing to attempt to elucidate the mechanism

of inhibition of viral production by EGCg. The protease kinetics experiment using a quenched fluorogenic substrate was performed for evaluation of both catechin isomers. EGCg inhibited the protease activity continuously at relatively high concentrations, greater than 10 μM, and the inhibition occurred in a dose-dependent manner. Although 100 μM EC also inhibited protease, the activity was weak and transient at the early stage of the reaction with no continuous inhibition. This may be due to non-specific interference, since the reaction level of the positive controls was reached and no significant inhibition was observed at concentrations lower than 50 μM (data not shown).

The inhibitory effects of EGCg or EC on chronically infected cells were monitored for 14 days (Fig. 7). The inhibition of viral production by EGCg was 40–50% greater than that by EC at the same concentration. Based upon this finding, plus the results of the protease inhibition experiment, the galloyl group is deemed indispensable for the biological activities of EGCg, which is consistent with previous reports on the anti-bacterial effects.

In conclusion, the effects of EGCg on several segments of the HIV life cycle were examined, and our studies demonstrated that virion-destruction and inhibition of RT were responsible for decreased viral activity. In chronically infected cells, EGCg inhibited viral production at higher concentrations than were used for acute infection, and viral regulation was partially inhibited during the LPS-stimulated state, which is mediated by the NF-κB pathway. Similarly, anti-protease activity was observed in cell free assays. These results were only observed with monocytoid host cells. The monocyte is an important target cell of HIV during its initial infection and is well known as a reservoir during the latent stage. In this study, we hypothesized that the effects observed on monocytoid cells might depend on phagocytosis of the EGCg. The details of the mechanism, however, are still unclear due to differences between monocytes and T-cells in their cell surface receptors, their responses to several stimulators, multi-drug resistance (MDR) pumps, and behavior during viral infection. We are now preparing to evaluate combination treatment using EGCg with other *anti*-HIV drugs based on the findings that EGCg has some potency for enhancement of the biological effects of antibiotics and prohibit the emergence of drug resistance in combination treatments in anti-bacterial studies. We are also planning to perform subsequent studies utilizing liposomal EGCg with the aim of introducing low-toxicity, direct-target therapy (Flasher et al., 1994; Schreier et al., 1994).

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